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14. ABSTRACT

The key objective of this project is to define a resistance mechanism for treating ER^+ breast cancer by endocrine therapy, such as tamoxifen. This project was guided by a clinical study searching for driver mutations that promote resistance to tamoxifen, leading to the discovery that inactivating NF1 (neurofibromin) is a key factor driving resistance. This project investigates the hypothesis that inactivating NF1, previously best known as a negative regulator for Ras by acting as a GAP (GTPase Activating Protein), can not only activate Ras, but also ER by interacting with ER's co-regulators. This is a collaboration between two PIs with complementary expertise. Dr. Chang is a molecular biologist and responsible for Aim 1, which is to investigate NF1's interaction with known ER co-regulator. Dr. Ellis is a physician scientist who is responsible for Aim 2 to establish a treatment strategy to treat NF1-deficient ER^+ breast cancer. We have made great stride in this project period from both aims. Briefly, we have uncovered a surprising GAP-independent activity of NF1 that it is also a co-repressor for ER. This is backed by technologically sophisticated RNA-seq and ChIP-seq experiments. As such, inactivating a single tumor suppressor NF1 can activate two powerful oncogenic pathways, which must be co-targeted for effective treatment. To this end, we demonstrate that using patient-derived xenograft model that this can be achieved by FDA-approved fulvestrant in combination with dabrafenib and trametinib.

15. SUBJECT TERMS
NF1, Neurofibromatosis type 1, Ras GTPase, GAP, Neurofibromin, estrogen receptor, gene expression, targeted therapy, ER co-regulators, ER-co-repressors.

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INTRODUCTION

While many targeted therapies are available for breast cancers that express estrogen receptor-α (ER^+), relapse and death is common and closely linked to resistance to these ER-targeting agents. The overall **objectives** of this project are to define mechanisms of endocrine therapy resistance, and to design etiology-matched treatments to improve outcomes. This project investigates the **hypothesis** that NF1 (Neurofibromatosis type 1), previously known chiefly as a negative regulator for Ras as a GAP (GTPase Activating Protein), also negatively regulates ER signaling via co-activator interactions, such that NF1 loss in tumors leads to aggressive tumor behavior not only through activated Ras signaling but also through increased ER activity. This project is a collaboration between Eric Chang (the initiating PI) and Matthew Ellis (Partnering PI), and they each contribute equally to this project (see below).

KEYWORDS

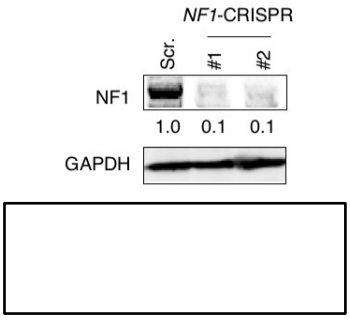
NF1, Neurofibromatosis type 1, Ras GTPase, GAP, Neurofibromin, estrogen receptor, gene expression, targeted therapy, ER co-regulators, ER-co-repressors.

ACCOMPLISHMENTS

To investigate our hypothesis, this proposal has two specific aims: In **Aim 1** we are defining how NF1 regulates expression of ER target genes by investigating a direct interaction between NF1 and canonical ER transcriptional co-regulators, with a particular focus on how NF1 may inhibit activities of co-activators. This is the main responsibility of the Chang lab. **Aim 2** is the main responsibility of the Ellis lab, with the goal of establishing a strategy to effectively treat NF1-deficient ER^+ breast cancers by rationally combining anti-Ras and anti-ER approaches. We have made great strides in the studies of both aims, and a revised manuscript describing our findings (see "Products" and the submitted manuscript attached as an Appendix) was submitted to the high impact journal *Nature*. Our task-specific progress is as follows:

<u>Major Task 1</u> (responsibility of the Chang lab): To define how NF1 regulates expression of ER target genes by investigating a direct interaction between NF1 and canonical ER transcriptional co-regulators.

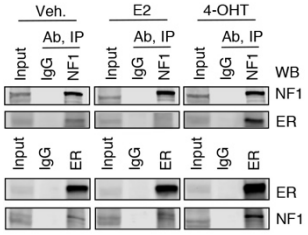
Subtask-1: Build vectors needed for this project. In addition to gene silencing using shRNA, which was generated in the last reporting period, we have now generated two NF1 knock-out cell lines using CRISPR (Fig. 1). These cell lines behave the same as cells carrying the shRNA that will be shown below.



Subtask-2: Measure effects of NF1 on half-lives of ER and co-activator SRC-1. We have determined that when *NF1* expression was silenced, no major change of steady state protein levels of ER and SRC-1. Thus, regulating ER and SRC-1 stabilities are unlikely to be the target of NF1 and will not be pursued further.

Subtask-3: Measure NF1-dependent nuclear trafficking of ER and SRC-1. In the last project period, we have shown that while more ER and SRC-1 are found in the nuclei upon E2 (estradiol) stimulation, NF1 loss increases this further. We have decided to put the study of trafficking on hold to focus on what appears to be a more important question: what happens to ER once it is in the nucleus of NF1-depleted cells.

Subtask-4: Measure NF1 and ER binding. In the last project period, we have detected the binding using the mammalian two-hybrid system, as well as using purified components *in vitro*. In this project period we have focused on measuring binding between endogenous proteins and whether it is ligand dependent. We have data using MCF7 cells that ER antibody can co-immunoprecipitate (co-IP) NF1 and NF1 antibody can co-IP ER, and that this interaction is stimulated by an ER antagonist (4-OHT) but not by E2 (Fig. 2). This suggests that ER anatagonists may function by recruiting NF1 to attenuate ER activity.



Subtask-5/6: Measure NF1-dependent co-regulator recruitment to the ERE (Estrogen Responsive Element) by a cell-free system. In the last project period, we have used a cell-free ERE-bead pull-down assay to show that NF1, as well as another co-repressor, HDAC1, is recruited to the ER-ERE complex in the presence of 4-OHT, but not E2. Together with the findings above that the binding between NF1 and ER is also stimulated by 4-OHT, it strongly suggests that NF1 has transcription co-repressor activity. We have thus pursued this more thoroughly by performing RNA-seq experiments to analyze how loss of NF1 impacts gene expression in an unbiased fashion. As shown in Fig 3A, we performed RNAseq to profile differential gene expression in $NF1^{+}$ vs $NF1^{kd}$ cells after E2 stimulation and found that 72% of well-known E2-responsive genes seen in $NF1^{+}$ cells were also seen in E2-stimulated $NF1^{kd}$ cells (Fig. 4A). Similar results were detected in patients (not shown). Importantly, the majority of E2-induced genes in $NF1^{+}$ cells are more strongly *induced* in *NF1*^{kd} cells; conversely, E2-repressed genes are more strongly *repressed* in *NF1* ^{kd} cells.

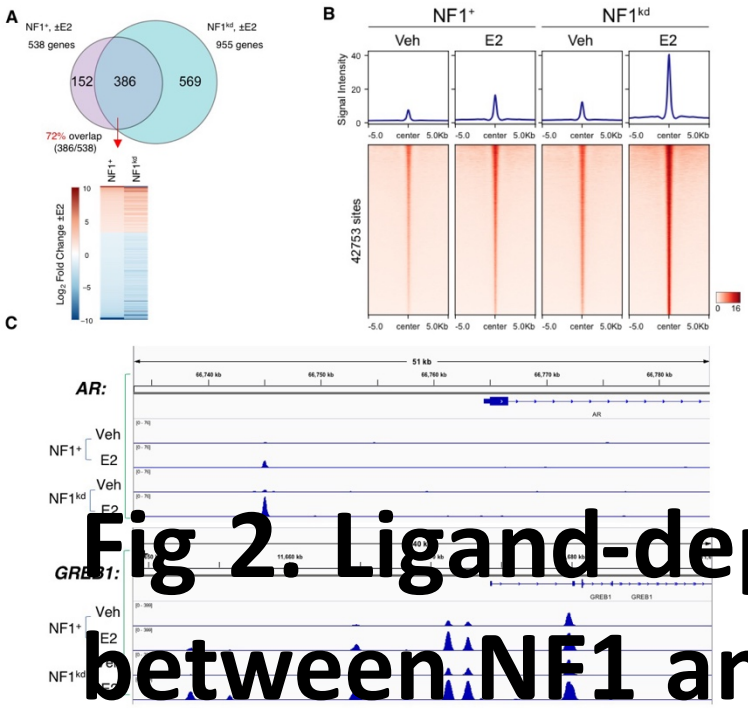
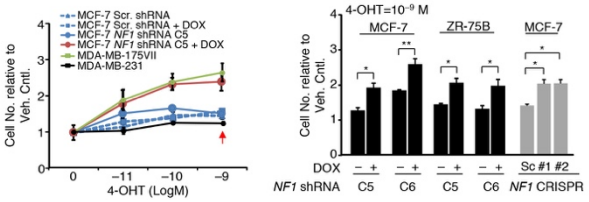


Fig 2. Ligand-dependent co-IP between NF1 and ER in MCF7 cells.

Subtask-7: Measure ER ChIP [illegible] to confirm binding of ERE or *AP-1* promoter. We have gone beyond the original scope and conducted an ER ChIP-seq experiment which shows that NF1-depletion promotes ER recruitment to the chromatin. As expected the ER binding sites are highly enriched by ERE sites (not shown). There is a 77% overlap between the genes identified in the RNAseq and ER ChIP-seq experiments (not shown), suggesting that changes in expression of the genes seen in the former are mostly due to direct ER recruitment to the EREs.

Subtask-8: Repeat above with more cell lines. We have repeated key experiments using several cell lines and will show one figure here (Fig 4) to illustrate that NF1 depletion induces

tamoxifen agonism such that tamoxifen can now stimulate cell growth instead of blocking it (more can be seen in the attached manuscript).



Subtask-9: Analyze ER-IP by MS and ChIP-seq. These experiments will be pursued in the future.

Subtask-10: Write a paper. We have now published in *Nature Communications* the original patient profiling study that inspired this project (below). Most of the results described here have been included in a revised manuscript submitted to *Nature* (see Appendix).

<u>Major Task 2</u> (Responsibility of the Ellis lab): To establish a strategy to treat NF1-deficient ER$^+$ breast cancers by rationally combining anti-Ras and anti-ER approaches.

Subtask-1/2: Seek antibody for IHC. We have generated a monoclonal antibody against NF1 and thoroughly tested it by immunofluorescence staining using several controls as requested by the reviewers of our paper, and the final results are shown in Fig 5. We have also made progress in the IHC. As shown in Fig 6, we can easily detect NF1 in T47D cells, which have the highest NF1 levels among the ER$^+$ cell lines we have tested (Fig 5), but no signal was seen in the NF1null MDA-MB-175 cells. However, the challenge now is that for cell lines that express low/medium levels of NF1, such as MCF7 cells, we also could not detect any signal. We thus need to further optimize the protocol to better control background/signal ratios.

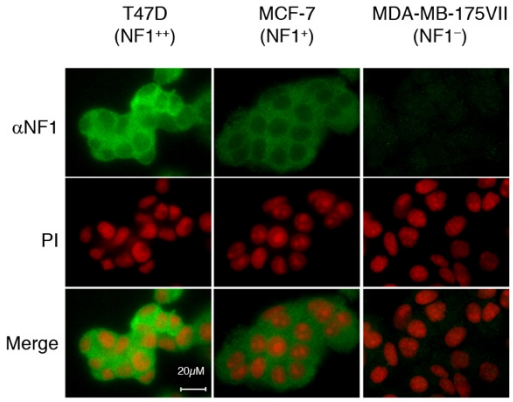
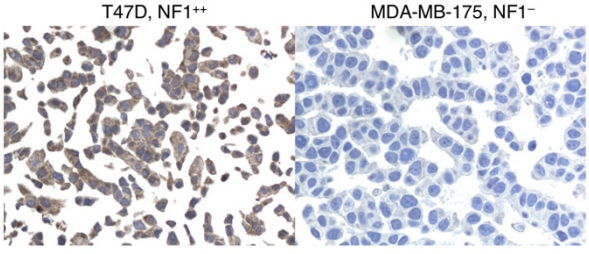


Fig 4. Tamoxifen in depleted NF1. The left shows cells seeded in tamoxifen (4-OHT), and (red arrow) of more cell lines exp two *NF1* shRNA on cells are "naturally" NF1-null due mer is ER$^+$ while the latter is ER$^-$

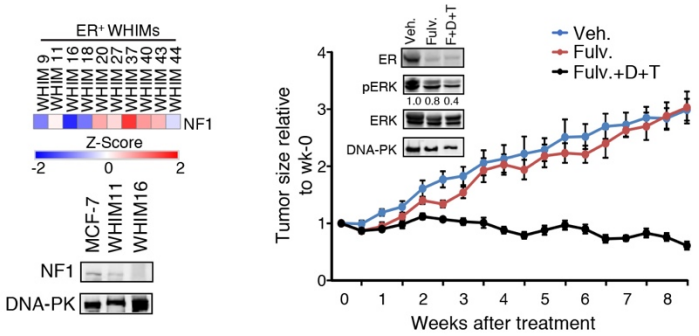
Subtask-3: Screening PDX with NF1 deficiency. We have identified an ER$^+$ NF1$^-$ PDX, WHIM16. WHIM16 was derived from a patient who died from drug-resistant disease and appropriate inhibitor treatments, so it represents a late-stage patient who had already acquired fulvestrant resistance. Indeed, as shown in Fig. 7, WHIM16 showed minimal response to fulvestrant single treatment. Remarkably, adding dabrafenib (D) and trametinib (T), which block the Raf and MEK downstream of Ras, dramatically inhibited tumor growth.



Subtask-4: Perform IHC to screen our original patient cohort to measure correlation between NF1 loss and relapse. This will be performed when IHC is ready.

Subtask-5: Treat cell lines with various drugs and measure cell growth *in vitro*. This *in vitro* study is mostly completed. Besides MCF7 cells as shown

in the previous project period, we have now also demonstrated the same results using ZR75 cells. All of these validate our hypothesis that in these NF1-deficient ER$^+$ breast cancer cells, a combination therapy using a SERD, e.g., fulvestrant, and anti-Ras agents, e.g., dabrafenib and trametinib, is needed for optimal treatment efficacy.



Subtask-6/7: Measure tamoxifen resistance and E2 hypersensitivity in mice: This has been accomplished in the last period using the MCF7 xenograft model.

Subtask-8: Test treatment strategy using cell lines in mice. This has been accomplished in the last period using the MCF7 xenograft model.

Subtask-9: Test treatment strategy using PDXs in mice. See above.

Subtask-10/11: Perform MIB-MS to identify the resistance mechanism in treated cell lines. This will be performed in the future.

Subtask -2: Evaluate data and repeat key experiments and write paper. See above, Task 1, Subtask 10.

We have disseminated these observations by (1) submitting a paper, (2) presenting as a talk in a meeting, and (3) applying and receiving additional funding — see "Products" below.

Fig 7. Treatment o

anti-Ras agents. Los

[5](left, bottom). The t

IMPACT

Despite the fact NF1 has been mostly known for its ability to negatively regulate Ras, the results of this project suggest that it is also a negative regulator for ER. Thus, inactivation of a single tumor suppressor can turn on two powerful oncogenic pathways, which currently are not being co-targeted for therapy. The results from this project strongly suggest that we must combine Ras pathway inhibitors and a SERD in order to effectively treat NF1-deficient cancers driven by ER, while tamoxifen may be contraindicated due to ER agonism.

In fact, a prospective treatment trial with this approach is currently being considered. The trial is a single arm multi-institutional phase II trial (Simon two-stage design) that will examine the efficacy of a combined therapy approach, trametinib + fulvestrant, in women with NF1-deficient ER^+ metastatic breast cancer. The trial has been developed into a working protocol with assistance from the AACR – "Methods in Clinical Research" meeting at Vail, CO 2017. Women with metastatic ER^+ breast cancer who have progression of disease on AI + CDK 4/6 inhibitors would be eligible for tumor profiling. If NF1 deficiency is found, they would be screened for this trial. The protocol for the trial will soon be submitted to the IRB for approval. This grant will directly impact this protocol by providing diagnostic strategies and the preclinical rationale.

CHANGES/PROBLEMS: Doug Chan was a mass spectrometry (MS) expert in the Ellis's lab with a proposed 2.4 Months effort/salary. He has since left, and his responsibility has been taken over by Dr. Beom-jun Kim. However during this funding period, we have focused more on animal work, and less so on MS. Therefore, we have reduced Kim's effort (to 1.8 Months), while adding support for an animal technician Purba Singh (1.2 Months), without greatly affecting the budget for personnel.

PRODUCTS:

(1) With support from this project, the original patient profiling study has been published in *Nature Communications*:

Obi Griffith, Nick Spies, Meenakshi Anurag, Malachi Griffith, Jingqin Luo, Dongsheng Tu, Belinda Yeo, Jason Kunisaki, Christopher Miller, Kilannin, Krysiak, Jasreet Hundal, Benjamin Ainscough, Zachary Skidmore, Katie Campbell, Runjun Kumar, Catrina Fronick, Lisa Cook, Jacqueline Snider, Sherri Davies, Shyam Kavuri, Eric C. Chang, Vincent Magrini, David Larson, Robert Fulton, Shuzhen Liu, Samuel Leung, David Voduc, Ron Bose, Mitchell Dowsett, Richard Wilson, Torsten Nielsen, Elaine Mardis, Matthew J. Ellis. The prognostic effects of somatic mutations in ER-positive breast cancer, *Nature Communications*, in press.

(2) A revised manuscript describing the bulk of the results from this project has been peer reviewed by *Nature.* While the paper was returned, we are responding to the comments and will re-submit.

Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafaee, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis, Eric C. Chang. Neurofibromin is an Estrogen Receptor-α Transcriptional Corepressor in Breast Cancer. *Nature,* under peer review.

(3) Dr. Foulds is a key member of the team and was able to use the support of this grant to publish a paper focusing on profiling co-regulators associated with mutant ER:

Gates LA, Gu G, Chen Y, Rohira AD, Lei JT, Hamilton RA, Yu Y, Lonard DM, Wang J, Wang SP, Edwards DG, Lavere PF, Shao J, Yi P, Jain A, Jung SY, Malovannaya A, Li S, Shao J, Roeder RG, Ellis MJ, Qin J, Fuqua SAW, O'Malley BW, Foulds CE. Proteomic profiling identifies key coactivators utilized by mutant ERα proteins as potential new therapeutic targets. *Oncogene,* in press.

(4) This study was selected for a talk at the 2018 GeneMed meeting in Houston:

Ze-Yi Zheng, Meenakshi Anurag, Jin Cao, Burcu Cakar, Xinhui Du, Jing Li, Philip Lavere, Jonathan T. Lei, Purba Singh, Sinem Seker, Doug Chan, Xi Chen, Kimberly C. Banks, Richard B. Lanman, Maryam Nemati Shafaee, Susan Hilsenbeck, Charles E. Foulds, Matthew J. Ellis, Eric C. Chang. Neurofibromin is an Estrogen Receptor Alpha Transcriptional Corepressor in Breast Cancer. GeneMed, 2018.

(5) With the direct support of this DOD grant, a pre-application focusing on another aspect of NF1 biology in breast cancer has been selected for a full application for a grant available at Breast Cancer Research Foundation. We have also just been notified the award of another NF1-related grant from the Cancer Prevention and Research Institute of Texas (CPRIT). We have also been productive in other areas using the DOD funding which has led to the award of an R21 from NIH. This R21 focuses on how Ras may promote DCIS progression in breast cancer.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name	**Project role**	**ORCID ID**	**Person Mon worked**	**Project contribution**	**Funding support**
Eric Chang	PI	0000-0002-1375-5088	4	Design and execute all the studies in Aim 1, and write the paper.	This grant.
Matthew Ellis*	Partnering PI	0000-0002-8467-8534	0.6	Design and execute the studies in Aim 2, and write the paper.	This grant.
Charles Foulds	Co-Investigator	0000-0003-4908-1473	0.6	Assist in the design and execution of the studies in Aim 1, and the study of the co-regulator function in particular,	This grant.

				and write the paper.	
Beom-jun Kim*	Co-Investigator	0000-0003-3109-8170	1.8	Assist Dr. Ellis in the studies in Aim 2.	This grant.
Purba Singh*	Tech	0000-0002-8467-8534	1.2	Conduct animal studies under the direction of Dr Ellis in Aim 2	
Zeyi Zheng	Staff Sci	0000-0001-6536-4874.	12	Assist Dr. Chang in the design and execution of all the studies in Aim 1, and supervise Ms Nguyen.	This grant
Tiffany Nguyen	Tech	NA.	12	Provide technical support on all projects.	This grant

*Peronnel supported by Ellis's protion of the grant.

SPECIAL REPORTING REQUIREMENTS

None.

APPENDIX:

The revised paper submitted to *Nature.*

Neurofibromin is an Estrogen Receptor-α Transcriptional Co-repressor in Breast Cancer

(Running title: NF1 is an ER Co-repressor)

Ze-Yi Zheng[1], Meenakshi Anurag[1], Jin Cao[2], Purba Singh[1], Jianheng Peng[1,3], Jonathan T. Lei[1,4], Nhu-Chau Nguyen[1], Philip Lavere[2], Jing Li[1], Xin-Hui Du[1,5], Burcu Cakar[1], Wei Song[1], Beom-Jun Kim[1], Sinem Seker[1], Doug W. Chan[1,2], Guo-Qiang Zhao[5], Xi Chen[2], Kimberly C. Banks[6], Richard B. Lanman[6], Maryam Nemati Shafaee[1], Xiang H.-F. Zhang[1,2], Suhas Vasakair[1], Bing Zhang[1], Susan G. Hilsenbeck[1], Charles E. Foulds[2,7], Matthew J. Ellis[1,2,8†], Eric C. Chang[1,2†]

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[5]Department of Bone and Soft Tissue, Zhengzhou University Affiliated Henan Cancer Hospital and College of Basic Medical Sciences, Zhengzhou University, P. R. China.

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[8]Department of Medicine, Baylor College of Medicine, USA.

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Neurofibromin, encoded by the *NF1 N*euro*f*ibromatosis type *1* gene, is a GTPase-Activating-Protein (GAP) that attenuates Ras signaling[1]. However, extensive evolutionary sequence conservation beyond the GAP domain suggests other hitherto undefined functions. Herein we report a GAP-independent activity of NF1 in the context of endocrine therapy resistant estrogen receptor-α positive (ER^+) breast cancer. We find that *NF1*-silencing increases ER activity to cause estradiol (E2) hypersensitivity and tamoxifen agonism. NF1 nuclear levels and binding to ER are both enhanced by tamoxifen, but not by E2. The binding to ER is mediated by two consensus leucine/isoleucine-rich co-repressor motifs[2,3]. When conserved residues in these NF1 motifs are mutated, including a somatic event in an ER^+ breast cancer, ER binding and transcriptional repression are decreased without impairing GAP activity. Conversely, GAP mutations in NF1 do not impact its ER binding and repression. NF1-deficient ER^+ breast cancer models retain sensitivity to selective estrogen receptor degraders (SERDs), such as fulvestrant, and further targeting Raf/MEK suppresses acquired fulvestrant resistance. In conclusion, NF1 is a previously unrecognized ER co-repressor. Combining Ras pathway inhibitors and a SERD may be an effective treatment for NF1-deficient ER^+ breast cancers, while tamoxifen may be contraindicated due to ER agonism.

By sequencing tumor DNA from early stage ER^+ tumors treated with adjuvant tamoxifen monotherapy, we recently reported that nonsense (NS) and frameshift (FS) mutations in *NF1* are associated with a markedly higher risk of breast cancer recurrence and death (Griffith et al. *in press*). Furthermore, when samples of circulating cell-free tumor DNA from metastatic ER^+ breast cancer patients (n=535)[4] were sequenced, the

frequency of *NF1* mutation was dramatically higher (18%, Fig. 1a and Supplemental Table S1) than that in primary breast cancer (e.g., TCGA[5] at 2%, Fig S1a). Similar enrichment of *NF1* mutations in metastatic breast cancer has been reported by others[6,7]. These observations suggest that somatic *NF1* events are an important new class of progression mutation that may relate to treatment resistance.

NF1 NS/FS mutations may trigger mRNA degradation through nonsense-mediated decay. Consistent with this mechanism, greatly reduced full-length *NF1* mRNA levels were observed in samples from both TCGA (Fig. 1b) and METABRIC[8] (Fig. S1b) samples when NS/FS mutations were present. ER^+ breast cancer is also treated with aromatase inhibitors (AI), which lower E2 levels. We therefore examined gene expression data from the ACOSOG Z1031 clinical trial, where patients were treated with an AI before surgery[9]. These data demonstrated that while a multi-gene proliferation score (MGPS)[10] decreased after AI treatment in tumors with higher *NF1* mRNA levels, reduced suppression of MGPS was observed in tumors with low *NF1* mRNA levels, indicating AI resistance (Fig. 1c and Supplemental Table S2). Finally, many NS/FS mutations affect the C-terminus distal to the centrally-located GAP domain, and *NF1-R2450^{fs}* is the most frequent mutation of any *NF1* mutation type in the COSMIC database (Fig. S1c). We could not efficiently express NF1 missing a small portion of its C-terminus caused by the *NF1-R2450^{fs}* mutation or two other recurrent FS/NS mutations (Fig. S1c). These findings raise the possibility that depletion of the entire NF1 protein, rather than a stable truncated protein, is a common consequence of FS/NS mutations. A requirement for the loss of the entire NF1 protein for poor outcomes, and the lack of recurrent GAP-inactivating mutations in our sequencing studies[4], led us to speculate that NF1 may have additional functions besides Ras regulation.

To investigate the consequences of NF1 depletion on ER^+ breast cancer, MCF-7 ER^+ breast cancer cells were engineered to harbor lentiviruses expressing one of two doxycycline (DOX)-inducible shRNA clones (C5 and C6). Upon DOX addition (+DOX), NF1 protein levels were reduced by ~70%. Increased ERK phosphorylation versus vehicle treated (–DOX) or scrambled shRNA controls indicated the anticipated reduction in GAP activity (Fig. S1d). An NF1 shRNA-resistant expression construct reversed these effects (Fig. S1d).

Remarkably, when *NF1* expression was silenced (+DOX) in MCF-7 and ZR-75B cells, *in vitro* growth was stimulated by 4-hydroxy-tamoxifen (4-OHT) rather than inhibited, in comparison to the non-silenced control (–DOX) or the scrambled shRNA +DOX control (Fig. 1d and S1e), indicating a switch from 4-OHT antagonism to agonism. Furthermore, *NF1*-silenced cells proliferated at lower concentrations of E2 (10^{-13}M) than controls and higher E2 concentrations (>10^{-10}M) inhibited cell growth indicating E2 toxicity (Fig. 1e and S1f). Two pools of NF1 "knock-out" MCF-7 cells were independently created by CRISPR with results similar to the shRNA data (Fig. 1 d and e, and Fig. S1g). Finally, 4-OHT and E2 responses in ER^+ MDA-MB-175VII and ER^- MDA-MB-231 cells were also examined because these lines naturally harbor *NF1* frameshift mutations (+/pI679^{fs} and +/pY2285^{fs} in the former and +/pT467^{fs} in the latter)[11,12]. Both lines have lost all detectable NF1 protein by Western blotting (Fig. S1h). The ER^+ MDA-MB-175VII cells also exhibited 4-OHT agonism and E2 hypersensitivity and responded to E2 at even lower concentrations (e.g., growth stimulation at 10^{-15}M and E2 toxicity at >10^{-13} M) than shRNA-silenced MCF-7 and ZR-75B cells. The ER^- MDA-MB-231 cells were, as expected, unresponsive to 4-OHT or E2 indicating a requirement for ER for the endocrine

effects of NF1 perturbation (Fig. 1 d and e). E2 hypersensitivity and 4-OHT agonism were reproduced *in vivo* using an MCF-7-based xenograft mouse model: tamoxifen stimulated the growth of *NF1*-silenced tumors (Fig. 1f, left), and these tumors grew at a dose of E2 (0.05 mg dose) that had almost no discernable effect on the growth of control tumors (Fig. 1f, right).

Since the abnormal E2/tamoxifen responses observed in NF1-depleted cells are ER-dependent, we investigated whether NF1-depletion affects ER-dependent transcription. First, the mRNA levels of established ER-target genes were examined by qPCR in MCF-7 cells. The data showed that expression of *GREB1* and *pS2* in the presence of E2 was augmented by *NF1* shRNA (Fig. S2a) or CRISPR-mediated *NF1* knock-out (Fig. S2b). This enhancement was not due to an increase in ER levels (Fig. S2a) and could be reversed by the aforementioned shRNA-refractory NF1 construct (Fig S2a), indicating specificity. Gene expression was also stimulated by 4-OHT in MCF-7 cells depleted for NF1 by shRNA (Fig. S2c) or by CRISPR (Fig. S2b), consistent with conversion to agonism. E2 and 4-OHT-stimulated gene expression was similarly observed in *NF1*-silenced ZR-75B cells (Fig. S2d). Finally, we examined tumor tissues from the aforementioned MCF-7 xenograft model by qPCR and found that expression of ER target genes *GREB1* and *pS2* was greatly enhanced in *NF1*-silenced tumors (Fig. S2e).

To examine endogenous gene expression in a genome-wide fashion, RNA-seq. experiments were performed in control (–DOX) and *NF1*-silenced (+DOX) MCF-7 cells before and after E2 stimulation. Overall E2 altered expression of 538 in the control $NF1^{+}$ cells and 955 genes in $NF1^{kd}$ cells (Fig. 2a, and Table S3). There was an overlap of 386 genes between the two gene sets (Table S3) indicating that expression changes of 72%

(=386/538) of the observed E2-altered genes seen in $NF1^{+}$ cells were also altered in $NF1^{kd}$ cells. These overlapping genes, referred to as the "common E2-regulon," were analyzed by GSEA Hallmark pathway analysis and found to be highly enriched with well-established E2-responsive genes, such as *GREB1* and *pS2/TFF1*, which were detected separately as discussed above, as well as *PGR*, *PDZK1*, *NRIP1*, *AREG*, *EGR3*, *H19* and *FOS* (Fig. 2a and Table S3). Furthermore, nearly all genes upregulated after E2-stimulation in $NF1^{+}$ cells were more strongly induced in $NF1^{kd}$ cells, and the great majority of E2-repressed genes were also more strongly repressed, consistent with ER-hyperactivity upon NF1 depletion. The 417 (=955−538) E2-altered genes that were selectively observed in the NF1-depleted state (referred to as the "$NF1^{kd}$-unique E2-regulon") were also assessed by GSEA Hallmark Pathway analysis (Table S3). Aside from a predominance of additional E2-regulated genes, a K-Ras-dependent gene expression signature was also observed (Fig. 2a). Gene expression changes induced by 4-OHT were also examined by RNA-seq (Fig. 2b, and Table S3) and the results were similar to the E2-stimulated gene expression alterations confirming tamoxifen agonism in the NF1-depleted state. For example, the overlap of 4-OHT-altered gene expression between $NF1^{+}$ and $NF1^{kd}$ cells was 65% and was also highly enriched with well-established E2-responsive genes. Furthermore, genes that were induced by 4-OHT in $NF1^{+}$ cells were more highly induced, while 4-OHT-repressed genes were more strongly repressed in $NF1^{kd}$ cells.

To demonstrate whether the observed E2-induced gene expression patterns (Fig. 2a) could be replicated in patient samples, differentially expressed genes according to *NF1* status (with or without *NF1* NS/FS mutation) in the METABRIC and TCGA ER^{+} data sets were identified, and pathway enrichments were similarly assessed (Table S4). The results

were compared to the two MCF-7 E2-regulons described above. Supporting the conclusion that NF1 depletion dramatically affects the expression of E2-responsive genes in clinical ER^+ specimens, E2-responsive pathway terms were most significantly modulated by *NF1* FS/NS mutation status, followed by the K-Ras signature (Fig. 2c).

To determine whether NF1-mediated repression of ER occurs at the level of estrogen response element (ERE) activity, ER-ChIP qPCR experiments were performed. These studies demonstrated that ERE occupancy in the promoters of both *GREB1* and *pS2* was greatly enhanced by *NF1*-depletion (Fig. 2c). These data suggest that NF1 could function as a ligand-dependent co-repressor for ER. To investigate this postulate further, the motif homologies within NF1 were examined. Established ER co-repressors bind ER via their leucine/isoleucine-rich co-repressor motifs, and minor substitutions of L or I with an A can disrupt binding[16]. Interactions are also mediated by charge[3,13]. NF1 harbors two consensus co-repressor motifs designated here as M1 and M2 (Fig. 3a). In the UBC TAM series[14], a somatic M1 I417M mutation was noted. Additional mutations affecting key residues in M1 and M2 can be found in COSMIC and METABRIC and in neurofibromatosis type-1[15]. M2 is located in the GAP domain, which can be readily purified in *E. coli*[16]. A purified NF1 GAP domain strongly pull-down purified ER, confirming that the interaction between NF1 and ER is probably direct (Fig. 3b). A mammalian two-hybrid assay[17] was used to demonstrate that the ER-NF1 interaction can occur in intact cells. In these assays NF1 selectively bound to the ligand-binding domain of ER, but not the AF-1 domain (Fig. S3a), and the interaction was induced by 4-OHT, but not by E2, mimicking a known corepressor NCoR1 (Fig. S3b). No substantial interaction between NF1 and ER-β (Fig. S3c) was observed, indicating specificity. By generating a

monoclonal NF1 antibody for immunoprecipitation (Fig. S3d), we were able to perform reciprocal co-immunoprecipitation (Co-IP) experiments in MCF-7 (Fig. 3c) and ZR75B (Fig. S3e) cells and demonstrated that NF1 can co-immunoprecipitated ER and *vice versa*. In these Co-IP studies, interactions were stimulated by 4-OHT but not by E2 (Fig. 3c), supporting the conclusion that ligand-binding modulates the NF1-ER interaction.

A cell-free system[18] was used to confirm that ER ligands can impact NF1 recruitment to the ER-ERE complex. When purified ER, biotinylated EREs, and HeLa nuclear extract were combined, the E2-liganded ER-SRC-1-ERE complex could be pulled down by streptavidin beads[18] with little recruitment of NF1, mimicking the behavior of the co-repressor HDAC1, but NF1 and HDAC1 were recruited to the ER-ERE complex in the presence of 4-OHT (Fig. 3d). Using two separate polyclonal NF1 antibodies (Fig. S3f), we also demonstrated that endogenous NF1 was recruited to the promoters of *GREB1* and *pS2* (Fig. 3e) when 4-OHT was added, but not E2. In sum, these data support the hypothesis that NF1 is an authentic ER co-repressor.

To directly determine whether M1 and M2 are authentic ER-binding sites, L was substituted with A[16]. The I417M *NF1* somatic M1 mutant was also generated. The binding to ER (Fig. 3f), measured by the two-hybrid assay, was greatly reduced by both M1 and M2 mutations. The I417M M1 mutant and one M2 mutant were chosen to further demonstrate that repression of ER transcriptional activity was also weakened, as measured by an ERE-luciferase reporter assay[19] (Fig. S3g), while retaining full GAP activity (Fig. S3h). Conversely, two NF1 GAP inactivating mutants were identified to further demonstrate ER repression is GAP independent. These GAP mutants were identified in metastatic ER^+ breast tumors, NF1-R1362Q[4] and NF1-K1444R[20] (Fig. S3h). Both mutants

bound ER (Fig. 3g) and repressed ER (Fig. S3g) as efficiently as wild type NF1. One GAP and one M1 mutant were then chosen for further study by expressing them in *NF1*-silenced cells to levels comparable to NF1 in control cells (Fig. 3h). The enhanced *GREB1* and pS2 expression in *NF1*-silenced cells was repressed by wild type NF1 and the GAP mutant, but not by the M1 mutant (Fig. 3h). Furthermore, enhanced ERE-mediated gene expression was not restored to normal levels by the Raf and MEK inhibitors dabrafinib and trametinib (Fig. S3i). Together these data demonstrate that GAP activity and ER repression are functionally independent.

The human genome contains up to 14 Ras GAPs[21], which share no significant sequence homology beyond the GAP domain. For example, in p120GAP/RASA1, there is no identifiable M1; nor was there an M2 in a published GAP domain alignment study[22] (Fig. S3j). In the ER-NF1 Co-IP experiment shown above, ER did not interact with p120/RASA1, indicating specificity (Fig. S3e). Since protein co-expression is a strong predictor for co-functionality[23], the molecular activity classes of proteins whose levels positively correlated with those of NF1 in >100 patient derived samples were explored in the Clinical Proteomic Tumor Analysis Consortium (CPTAC) breast cancer database[24]. Remarkably, NF1 protein levels highly correlated with a number of transcription factor functionalities, and the "ligand dependent nuclear receptor binding" protein class in particular. p120GAP was similarly examined as a control, with mostly negatively correlations with these factors (Fig S3k).

For transcriptional regulation, NF1 must access the nucleus. While NF1 is mainly cytoplasmic, nuclear NF1 has been previously reported in a variety of cell types including ER^+ breast cancer cells[25-31]. In one study, NF1 nuclear localization in neuronal cells was

shown to be dependent on the Ran GTPase[31]. This mechanism may also operate in ER^+ breast cancer, because NF1 co-immunoprecipitated with Ran in MCF-7 cells (Fig. S3e). To further investigate how NF1 nuclear localization is controlled in ER^+ breast cancer cells, an immunostaining protocol was developed using a set of cell lines with varying degrees of NF1 (Fig. S1h), including the NF1 null-like MDA-MB-175 cells as a negative control (Fig. 4a). Nuclear NF1 levels in at least two ER^+ breast cancer cell lines could be increased by leptomycin-B (LMB), which blocks CRM1-dependent nuclear export, as shown by both microscopy (Fig. 4b) and cell fractionation experiments (Fig. 4c). Importantly, in cell fraction experiments NF1 nuclear levels were decreased by E2 and increased by 4-OHT indicating that hormone-regulated nuclear concentration is an aspect of the co-repressor role of NF1 (Fig. 4d). Overall these results suggest that while NF1 is mostly cytoplasmic at steady state, it is shuttled in and out of the nucleus, possibly by Ran and CRM-1, in a ligand-regulatable manner.

The clinical and functional data presented thus far suggests that NF1-deficient ER^+ breast tumors will not be effectively treated with tamoxifen or AI. However, when NF1 was depleted by either shRNA or CRISPR, the resulting MCF7 cells were still sensitive to the clinically-approved SERD fulvestrant (Fig. S4a). Similar observations were made in $NF1^{kd}$ ZR75B cells (Fig. S4b). MCF7 $NF1^{kd}$ cells are also sensitive to the experimental SERD AZD9496[33] (Fig. S4c). However, in *NF1*-silenced cells fulvestrant produced an enhanced compensatory activation of the Ras-Raf-MEK-ERK pathway which may promote cell survival and/or drug resistance in spite of effective ER inhibition (Fig. 5a). Thus, dual targeting to inhibit both ER and the Ras-Raf pathway was assessed to determine if NF1-deficient ER^+ breast cancer would be effectively treated with this combination. *In*

vitro the combination of dabrafenib and trametinib enhanced fulvestrant activity by greatly increasing apoptosis in $NF1^{kd}$ MCF-7 cells (Fig. 5b), an effect also observed for selumetinib (Fig. S4d) and AZD9496 (Fig. S4e). $NF1^{kd}$ ZR-75B cells were similarly more sensitive to the fulvestrant, dabrafenib, and trametinib combination (Fig. S4f). *In vivo,* while tumors from the MCF-7 $NF1^{kd}$ xenograft model initially responded to fulvestrant, at later time points these tumors acquired resistance. This fulvestrant resistant growth phase was blocked with dabrafenib and trametinib (Fig. 5c and S4g). In addition, we examined RNA-seq[20] and mass spectrometry data[34] to identify a patient-derived xenograft (PDX), WHIM16[20], with no detectable NF1 protein and very low levels of *NF1* mRNA (Fig. 5d). WHIM16 was derived from patient who died after the development of resistance to multiple lines of endocrine treatment including fulvestrant. As such, WHIM16 represents a late stage metastatic disease with pan-endocrine therapy resistance. Consistent with this history, WHIM16 showed minimal response to fulvestrant alone; however, dabrafenib and trametinib, together with fulvestrant, effectively inhibited xenograft growth (Fig. 5d and S4h).

In conclusion, this study presents multiple lines of evidence that NF1 is a transcriptional co-repressor of ER in ER^+ breast cancer in a manner that is independent of GAP activity. As a result, when NF1 is depleted through somatic mutation, ER function is enhanced, leading to tamoxifen agonism, E2 hypersensitivity/AI resistance, and poor outcome (Fig. 5e). In experimental systems, the tumor promoting effects of NF1 loss can be opposed with a SERD, but Ras activation can cause acquired SERD resistance, which could be effectively inhibited by drugs blocking Ras-Raf signaling. Further research may connect the ER hyperactivity induced by NF1 depletion to other paradoxical or

unexplained observations in ER^+ breast cancer, for example, regression of endocrine therapy resistant ER^+ breast cancer with E2 or synthetic estrogen treatment[35]. Additionally, the role of NF1 as an ER co-repressor may underlie the sexually dimorphic features of neurofibromatosis, including the preponderance of optic chiasm gliomas during female puberty[36].

Figures and figure legends

Figure 1

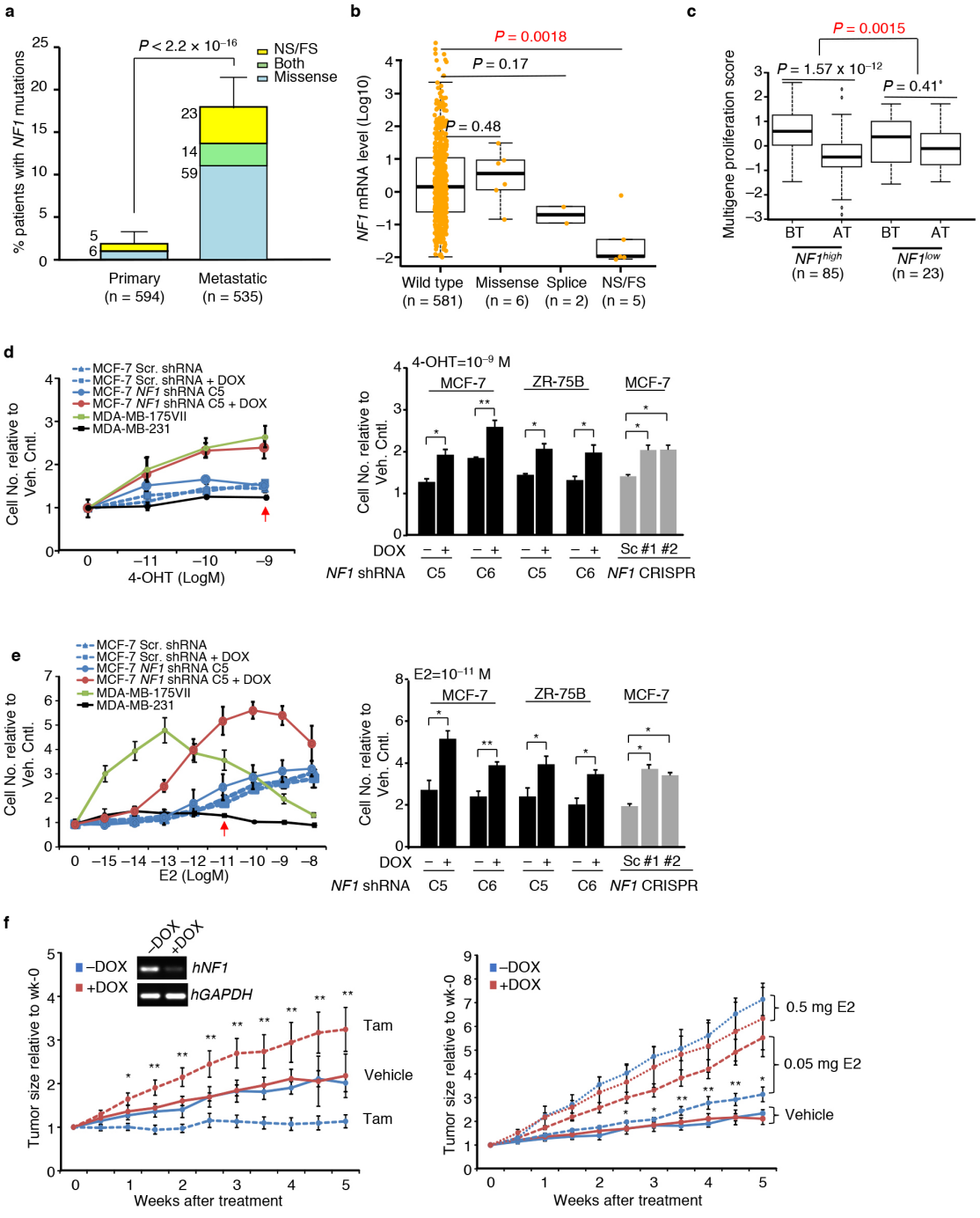
a
P < 2.2 x 10−16
NS/FS
Both
Missense
% patients with NF1 mutations
Primary (n = 594)
Metastatic (n = 535)
b
P = 0.0018
P = 0.17
P = 0.48
NF1 mRNA level (Log10)
Wild type (n = 581)
Missense (n = 6)
Splice (n = 2)
NS/FS (n = 5)
c
P = 0.0015
P = 1.57 x 10−12
P = 0.41
Multigene proliferation score
BT
AT
NF1high (n = 85)
NF1low (n = 23)
d
MCF-7 Scr. shRNA
MCF-7 Scr. shRNA + DOX
MCF-7 NF1 shRNA C5
MCF-7 NF1 shRNA C5 + DOX
MDA-MB-175VII
MDA-MB-231
Cell No. relative to Veh. Cntl.
4-OHT (LogM)
4-OHT=10−9 M
MCF-7
ZR-75B
DOX
NF1 shRNA
C5
C6
Sc #1 #2
NF1 CRISPR
e
E2 (LogM)
E2=10−11 M
f
–DOX
+DOX
hNF1
hGAPDH
Tam
Vehicle
Tumor size relative to wk-0
Weeks after treatment
0.5 mg E2
0.05 mg E2

Figure 1. NF1 loss promotes tamoxifen agonism and E2 hypersensitivity leading to poor patient outcome in ER$^+$ breast cancer. a, The portions of ER$^+$ primary vs. metastatic breast cancers carrying *NF1* mutations (Supplemental Table S1) were analyzed by Fisher Exact test. The number of patients carrying a particular type of *NF1* mutation is shown at the top of each column. **b,** Boxplot to analyze *NF1* mRNA levels in ER$^+$ breast tumors carrying different *NF1* mutations in the RNA-seq database of TCGA. *P*-value by Wilcoxon rank sum test. **c,** Patients were stratified by *NF1* mRNA levels according to TCGA definitions of high versus low expression (Mean – 1.5 × SD). Boxplot shows comparison of multigene proliferation score (MGPS) in tumors before treatment (BT) and after treatment (AT) by AI. The differences in MGPS before and after treatment in each NF1 group were analyzed by the Wilcoxon signed-rank test. The differences in MGPS as a result of treatment between the two *NF1* groups were further analyzed by Wilcoxon rank sum test (*P*-value marked in red). **d,** MCF-7 cells stably transfected with doxycycline (DOX)-inducible scramble or *NF1* shRNA (C5), as well as MDA-MB-231 and MDA-MB-175VII cells, were seeded in E2-deprived medium, to which 4-OHT at indicated concentrations was added (left). Cell numbers were measured 6 days later. The results (see also Fig. S1 e and g) from the cells treated with 4-OHT at a given concentration (red arrow) normalized to vehicle-treated control were quantified (right). n = 3 experiments, except for MCF-7 cells carrying *NF1* shRNA C5 (n = 6 experiments). **e**, Cells treated with E2 were similarly analyzed as in panel-d (see also Fig. S1 f and g). n = 3 experiments, except for MCF-7 cells carrying *NF1* shRNA C5 (n = 8 experiments). **f,** MCF-7 cells carrying DOX-inducible *NF1* shRNA were transplanted into the mammary fat pads of ovariectomized nude mice, supplemented by an E2-capsule. When tumors appeared, the original E2-

capsule was removed, and the resulting mice randomized into two sets (DOX or vehicle treated). Each set was then treated by either tamoxifen (left), or E2 (right). For *NF1*$^{+}$ tumors, n=10, 12, 12, and 8 mice per group for treatment of vehicle, 0.05 mg E2, 0.5 mg E2, and tamoxifen; for *NF1*^{kd} tumors, n = 10, 13, 11, and 8 mice per group. The inset shows *NF1*-silencing validation by qPCR 2 weeks post-DOX addition. The tumor size was measured twice weekly and plotted as the ratio relative to the initial tumor size right before drug treatment.

Figure 2

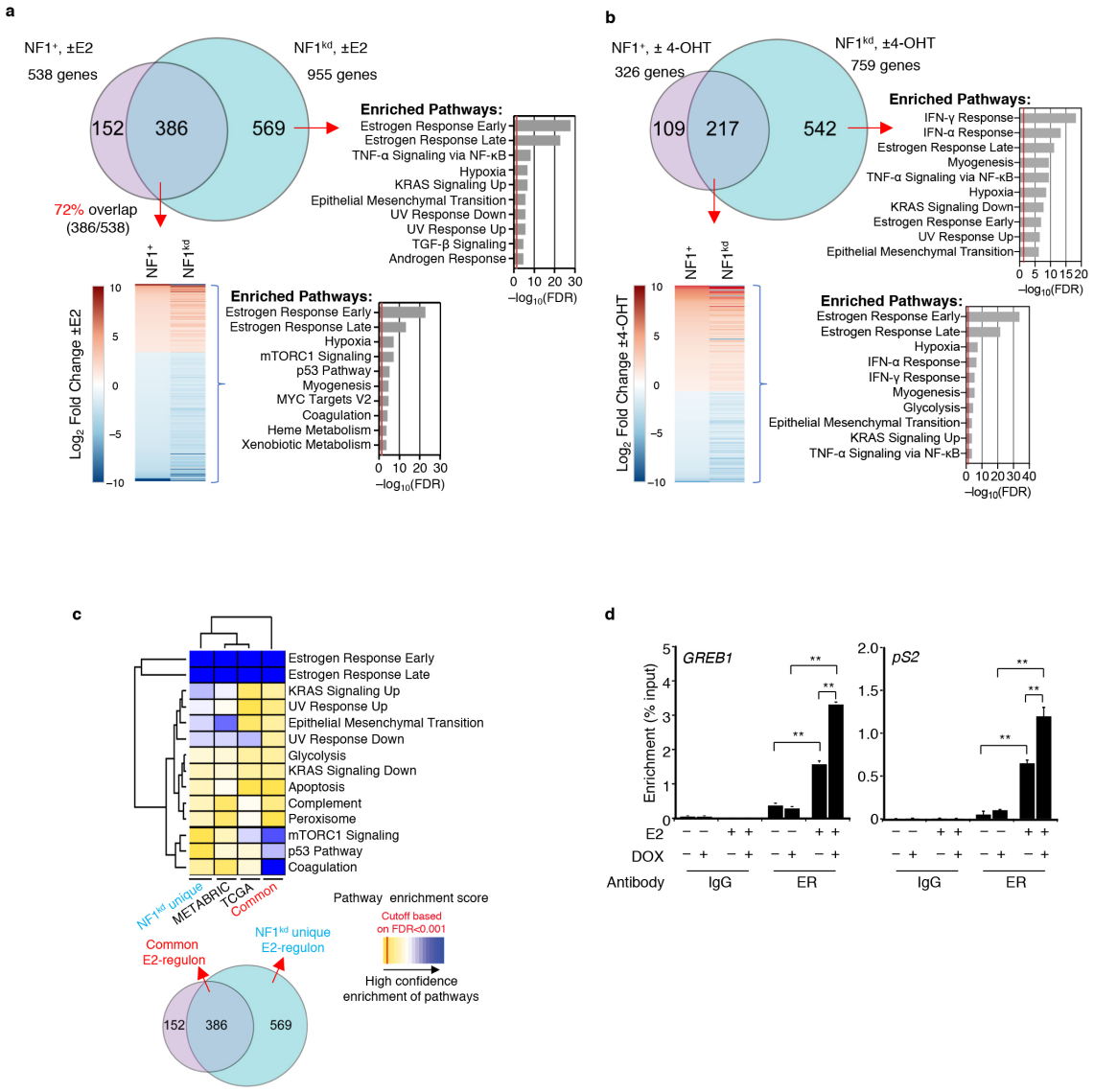

Figure 2. NF1 depletion globally enhances ER transcriptional activity. a, RNA-seq was performed on NF1^{+} or NF1^{kd} MCF-7 cells treated with E2 or vehicle. A Venn diagram depicts the number of E2-mediated differentially expressed genes in NF1^{+} (purple) vs. NF1^{kd} cells (teal) and those that overlap ("Common E2-regulon"). The latter in NF1^{+} and NF1^{kd} cells were ranked by (log2) fold-change in gene expression, and the Hallmark pathways enriched in this set were revealed by GSEA (red line marks FDR cutoff at 0.05).

GSEA analysis was also performed to similarly reveal Hallmark pathways enriched selectively in $NF1^{kd}$ cells ("$NF1^{kd}$ unique E2-regulon"). **b.** Gene expression changes stimulated by 4-OHT were similarly analyzed by RNA-seq as above. **c**, Genes identified in panel-2a were also examined in TCGA and METABRIC ER^+ breast cancer cases to identify those genes that are differentially expressed (Table S4) between tumors with wild type *NF1* and *NF1* FS/NS mutations. The enriched Hallmark pathways in patient data are presented along with the results of the two E2-regulons identified in MCF-7 cells to demonstrate high degrees of functional overlap similarity between cell line and clinical datasets. **c,** ER was immunoprecipitated from $NF1^+$ or $NF1^{kd}$ MCF-7 cells treated by E2 or vehicle, and ChIP-qPCR was performed to measure ER occupancy at the *GREB1* and *pS2* promoters. n = 2 experiments.

Figure 3

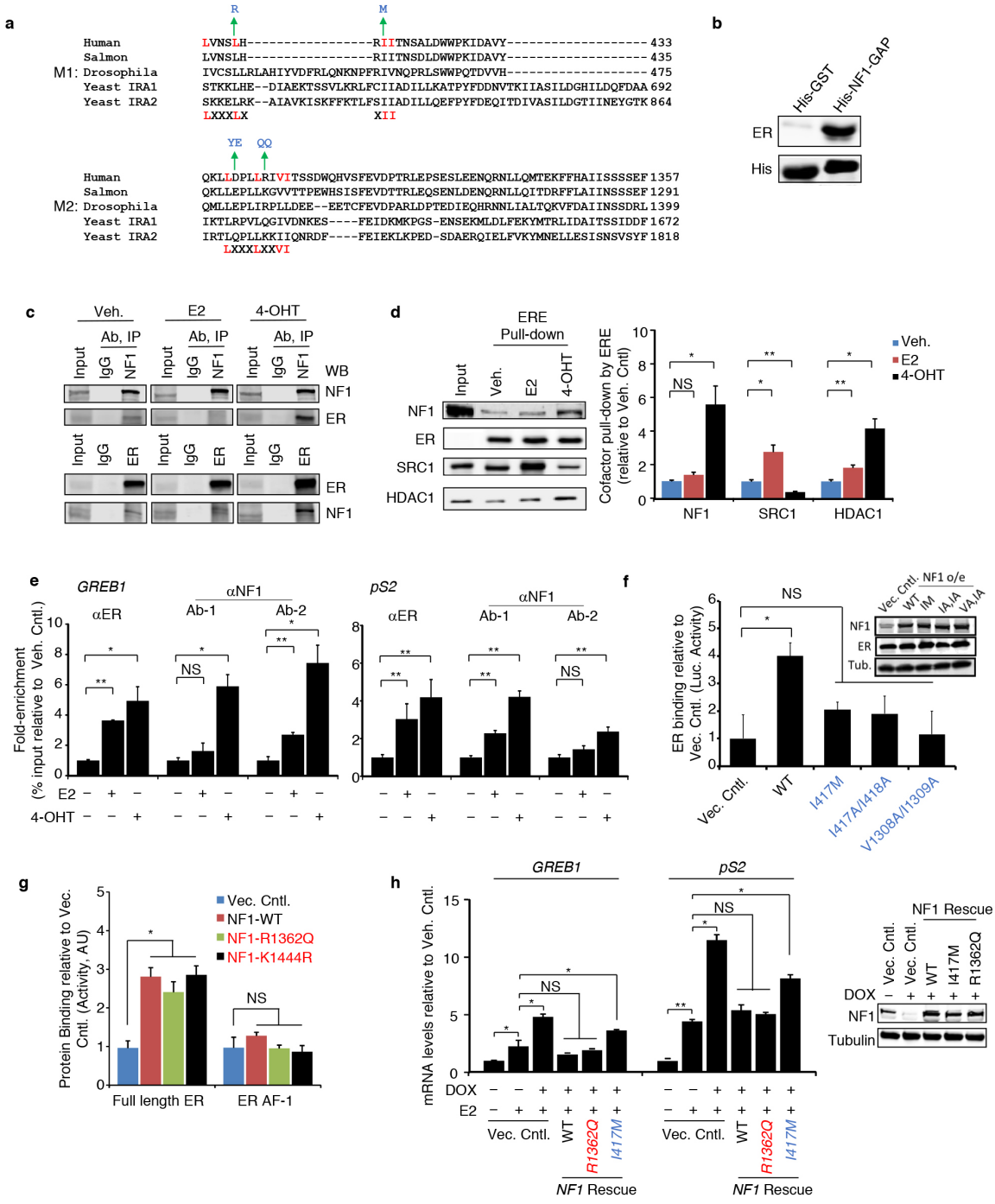

Figure 3. NF1 binds ER as mediated by co-repressor motifs and ER ligands. a, Protein alignment was created by ClustalW (MUSCLE). NF1 has two potential co-repressor

motifs, M1 and M2 (consensus sequences[3] shown at the bottom). Mutations found in cancers (COSMIC) or neurofibromatosis are colored blue. **b,** Purified ER was pulled-down by amylose beads containing bacterially expressed His-NF1-GAP domain, but not the control His-GST. **c**. MCF-7 cells grown in E2-deprived medium were treated with E2, 4-OHT or ethanol as vehicle, immunoprecipitated with NF1 or ER antibodies, and examined by Western blot. **d,** Indicated agents were added to the HeLa S3 nuclear extract together with purified ER, and pull-down experiments were conducted using ERE-immobilized beads. The resulting samples were analyzed by Western blot (left) and quantified (right). n = 3 experiments. **e,** NF1 or ER was chromatin-immunoprecipitated from MCF-7 cells stimulated by E2 or 4-OHT, and the associated chromatin was examined by qPCR using primers targeting the *GREB1* or *pS2* promoter. n = 2 experiments. **f**, The mammalian two-hybrid interaction was measured by luciferase activity and inset shows that wild type and mutant NF1 were expressed at the same levels in this assay. **g,** The two-hybrid interaction was similarly measured as above. n = 3 experiments. **h,** FLAG-tagged shRNA-refractory wild type or mutant NF1 was expressed in $NF1^{kd}$ MCF-7 cells to levels comparable to endogenous levels (right). qPCR was performed to measure *pS2* and *GREB1* expression (left).

Figure 4

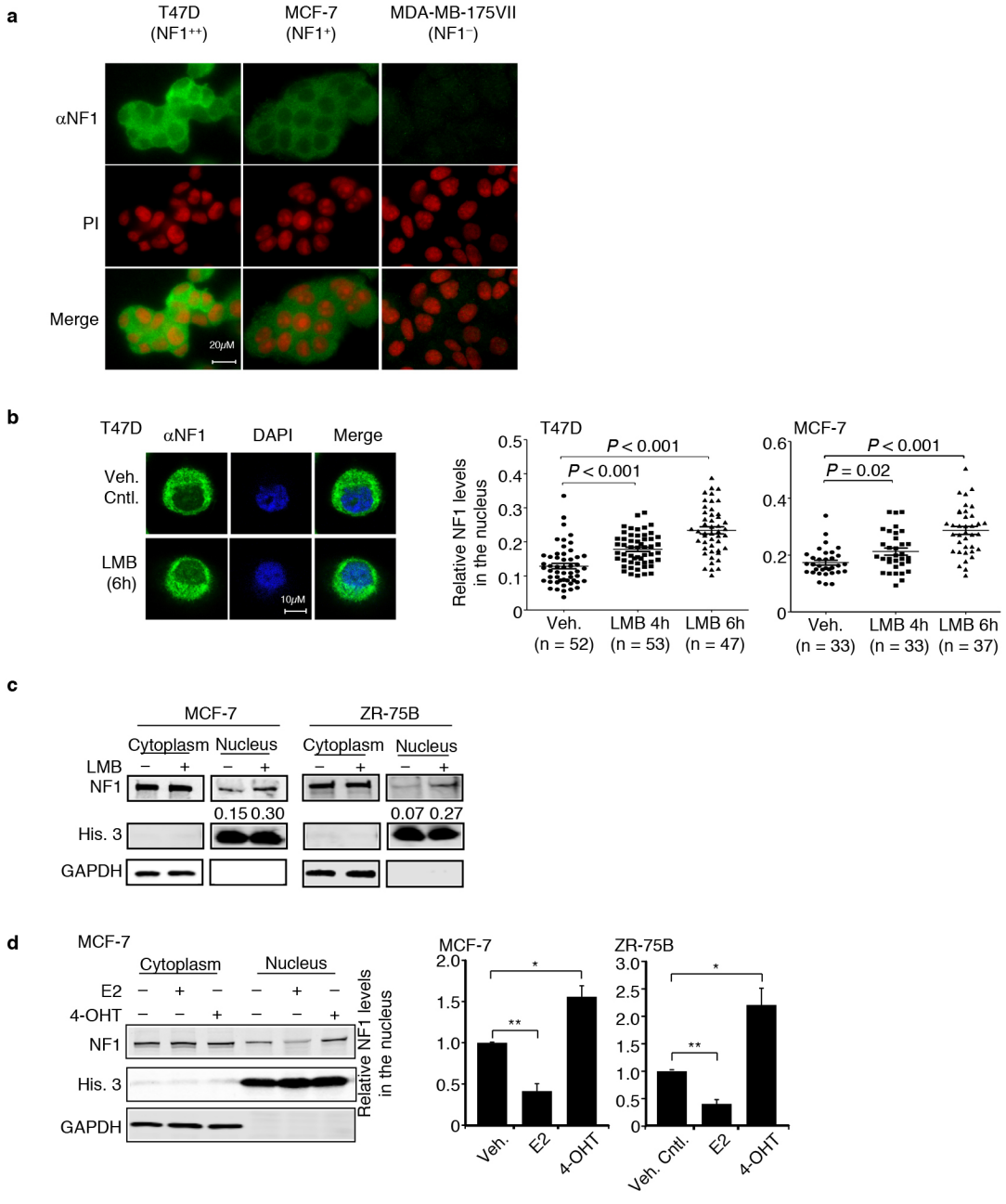

Figure 4. ER ligand-modulated nuclear accumulation of NF1. a, Immunofluorescence microscopy was performed on indicated cells with varying degrees of NF1 proteins, known by Western blot (Fig. S1h). PI marks the nucleus. **b**, T47D cells treated with LMB for 4 or

6 hrs were examined for NF1 localization by confocal microscopy (left), and the levels of NF1 the in nucleus vs. cytoplasm were quantified (see Methods section for detail) (middle). NF1 levels in MCF-7 cells were similarly quantified (right). n = number of cells quantified. **c**, Lysates from cells similarly treated with LMB for 6 hrs were separated into the nuclear and cytoplasmic fractions, marked by Histone-3 and GAPDH, respectively. NF1 levels in the nuclear vs. the cytoplasmic fraction were quantified. **d,** MCF-7 and ZR-75B cells after ligand stimulation were analyzed as in panel-c. n = 3 experiments.

Figure 5

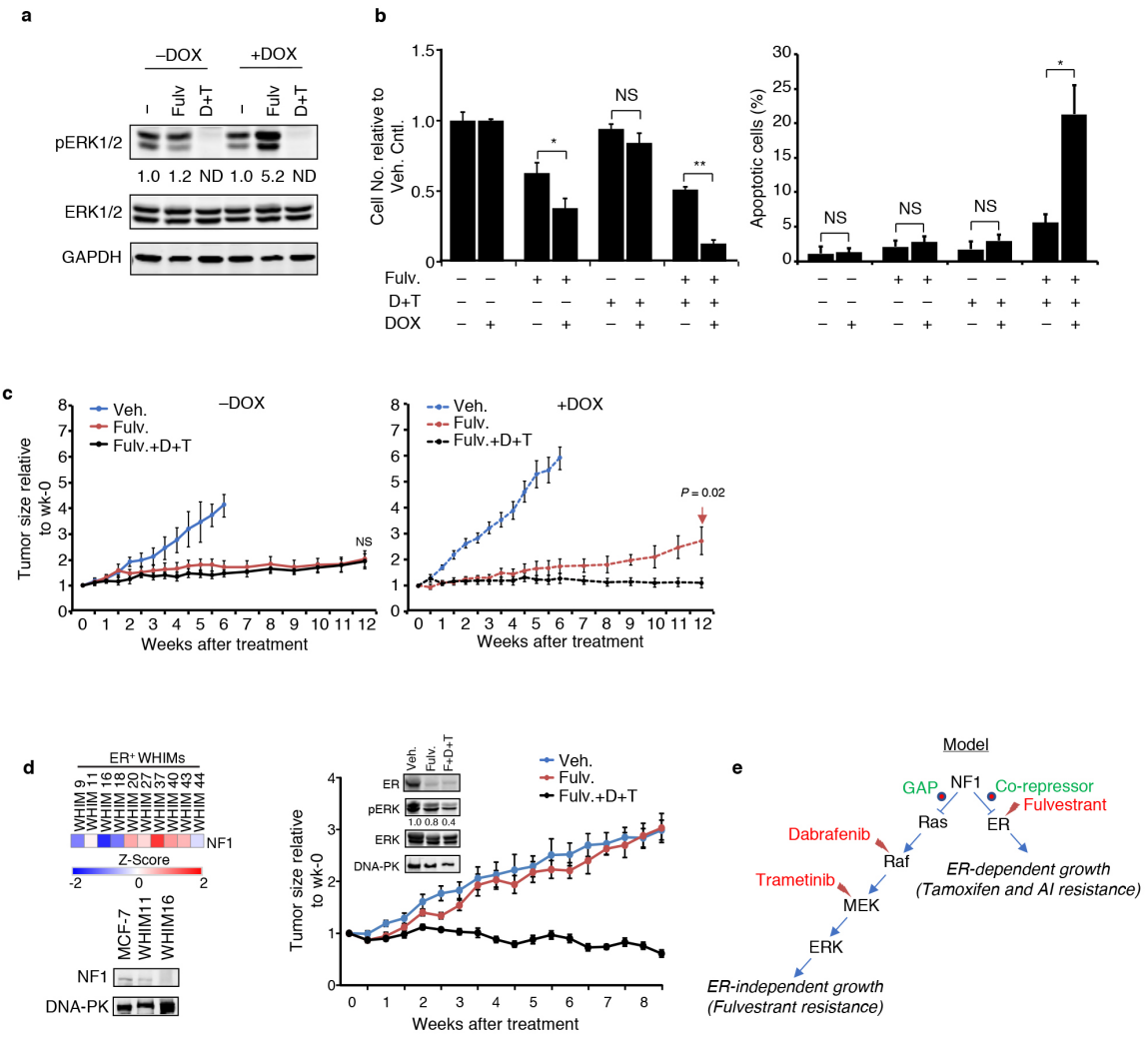

Figure 5. Co-targeting Ras and ER to treat NF1-deficient ER$^+$ breast cancer. a, Cells were seeded in 10^{-11} M E2 to which fulvestrant, dabrafenib, or trametinib were subsequently added at 10^{-9}, 10^{-6}, or 10^{-7} M, respectively. After 6 days, ERK phosphorylation was measured by Western blot, and phosphorylation levels relative to the vehicle-treated cells were set to 1. **b,** The same cells were treated by the same doses of indicated drugs as 5a. Cell numbers and apoptosis were measured 6 days later. We note that drug concentrations were selected (Fig. S4a) such that fulvestrant plus the dabrafenib

and trametinib combination can more easily produce an effect on the cells that is greater than either one group alone. n = 3 experiments. **c,** Xenograft experiment was performed similarly as in Fig. 1f, except that when tumors became palpable, the original E2 capsule was replaced by one that contains 0.05 mg E2, which can fully support the growth of $NF1^{kd}$ tumors (Fig. 1f). The resulting mice were then randomized (±DOX). All $NF1^{+}$ tumors had 13 mice per group. The vehicle-treated $NF1^{kd}$ tumors also had 13 mice per group but drug-treated groups had only 12 mice per group. Tumor growth was similarly measured and plotted as Fig. 1f. Tumor sizes at Week-12 of mice treated by fulvestrant alone and the 3-drug combination were compared to show that only toward the end of this experiment the former were becoming substantially bigger than the latter. **d**, Left, RNA-seq data from a panel of ER^{+} PDXs show that WHIM16 has very low *NF1* mRNA, and lack of NF1 protein was confirmed by Western blot using an antibody against human DNA-PK as a human-specific protein loading control. Right, tumor growth was similarly measured and plotted as above (n = 10 mice). One tumor from each group was analyzed by Western blot for treatment validation (inset). **e**, Model.

References

1 Yap, Y. S. *et al.* The NF1 gene revisited - from bench to bedside. *Oncotarget* **5**, 5873-5892, doi:10.18632/oncotarget.2194 (2014).

2 Hu, X. & Lazar, M. A. The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402**, 93-96, doi:10.1038/47069 (1999).

3 Heldring, N. *et al.* Structural insights into corepressor recognition by antagonist-bound estrogen receptors. *J Biol Chem* **282**, 10449-10455, doi:10.1074/jbc.M611424200 (2007).

4 Lanman, R. B. *et al.* Analytical and Clinical Validation of a Digital Sequencing Panel for Quantitative, Highly Accurate Evaluation of Cell-Free Circulating Tumor DNA. *PLoS One* **10**, e0140712, doi:10.1371/journal.pone.0140712 (2015).

5 TCGA. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70 (2012).

6 Yates, L. R. *et al.* Genomic Evolution of Breast Cancer Metastasis and Relapse. *Cancer Cell* **32**, 169-184 e167, doi:10.1016/j.ccell.2017.07.005 (2017).

7 Meric-Bernstam, F. *et al.* Concordance of genomic alterations between primary and recurrent breast cancer. *Mol Cancer Ther* **13**, 1382-1389, doi:10.1158/1535-7163.MCT-13-0482 (2014).

8 Curtis, C. *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346-352, doi:10.1038/nature10983 (2012).

9 Ellis, M. J. *et al.* Randomized phase II neoadjuvant comparison between letrozole, anastrozole, and exemestane for postmenopausal women with estrogen receptor-rich stage 2 to 3 breast cancer: clinical and biomarker outcomes and predictive value of the baseline PAM50-based intrinsic subtype--ACOSOG Z1031. *J Clin Oncol* **29**, 2342-2349, doi:10.1200/JCO.2010.31.6950 (2011).

10 Ellis, M. J. *et al.* Ki67 Proliferation Index as a Tool for Chemotherapy Decisions During and After Neoadjuvant Aromatase Inhibitor Treatment of Breast Cancer: Results From the American College of Surgeons Oncology Group Z1031 Trial (Alliance). *J Clin Oncol* **35**, 1061-1069, doi:10.1200/JCO.2016.69.4406 (2017).

11 Ogata, H., Sato, H., Takatsuka, J. & De Luca, L. M. Human breast cancer MDA-MB-231 cells fail to express the neurofibromin protein, lack its type I mRNA isoform and show accumulation of P-MAPK and activated Ras. *Cancer Lett.* **172**, 159-164 (2001).

12 Neve, R. M. *et al.* A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**, 515-527, doi:S1535-6108(06)00314-X [pii] 10.1016/j.ccr.2006.10.008 (2006).

13 Shiau, A. K. *et al.* The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927-937 (1998).

14 Nielsen, T. O. *et al.* A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 5222-5232, doi:10.1158/1078-0432.CCR-10-1282 (2010).

15 Xu, W., Yang, X., Hu, X. & Li, S. Fifty-four novel mutations in the NF1 gene and integrated analyses of the mutations that modulate splicing. *Int J Mol Med* **34**, 53-60, doi:10.3892/ijmm.2014.1756 (2014).

16 Bollag, G., McCormick, F. & Clark, R. Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. *EMBO J.* **12**, 1923-1927 (1993).

17 Chang, C. *et al.* Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. *Mol Cell Biol* **19**, 8226-8239 (1999).

18 Foulds, C. E. *et al.* Proteomic analysis of coregulators bound to ERalpha on DNA and nucleosomes reveals coregulator dynamics. *Mol Cell* **51**, 185-199, doi:10.1016/j.molcel.2013.06.007 (2013).

19 Hall, J. M. & McDonnell, D. P. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140**, 5566-5578, doi:10.1210/endo.140.12.7179 (1999).

20 Li, S. *et al.* Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. *Cell Rep* **4**, 1116-1130, doi:10.1016/j.celrep.2013.08.022 (2013).

21 Maertens, O. & Cichowski, K. An expanding role for RAS GTPase activating proteins (RAS GAPs) in cancer. *Adv Biol Regul* **55**, 1-14, doi:10.1016/j.jbior.2014.04.002 (2014).

22 Ballester, R. *et al.* The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**, 851-859 (1990).

23 Wang, J. *et al.* Proteome Profiling Outperforms Transcriptome Profiling for Coexpression Based Gene Function Prediction. *Mol Cell Proteomics* **16**, 121-134, doi:10.1074/mcp.M116.060301 (2017).

24 Mertins, P. *et al.* Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature* **534**, 55-62, doi:10.1038/nature18003 (2016).

25 Daston, M. M. *et al.* The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. *Neuron* **8**, 415-428 (1992).

26 Vandenbroucke, I., Van Oostveldt, P., Coene, E., De Paepe, A. & Messiaen, L. Neurofibromin is actively transported to the nucleus. *FEBS Lett.* **560**, 98-102, doi:10.1016/S0014-5793(04)00078-X (2004).

27 Li, C., Cheng, Y., Gutmann, D. A. & Mangoura, D. Differential localization of the neurofibromatosis 1 (NF1) gene product, neurofibromin, with the F-actin or microtubule cytoskeleton during differentiation of telencephalic neurons. *Brain Res. Dev. Brain Res.* **130**, 231-248 (2001).

28 Kweh, F. *et al.* Neurofibromin physically interacts with the N-terminal domain of focal adhesion kinase. *Mol. Carcinog.* **48**, 1005-1017, doi:10.1002/mc.20552 (2009).

29 Nousiainen, M., Sillje, H. H., Sauer, G., Nigg, E. A. & Korner, R. Phosphoproteome analysis of the human mitotic spindle. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 5391-5396, doi:10.1073/pnas.0507066103 (2006).

30 Beausoleil, S. A. *et al.* Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 12130-12135, doi:10.1073/pnas.0404720101 (2004).

31 Koliou, X., Fedonidis, C., Kalpachidou, T. & Mangoura, D. Nuclear import mechanism of neurofibromin for localization on the spindle and function in chromosome congression. *J. Neurochem.* **136**, 78-91, doi:10.1111/jnc.13401 (2016).

32 Kudo, N. *et al.* Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci U S A* **96**, 9112-9117 (1999).

33 Weir, H. M. *et al.* AZD9496: An Oral Estrogen Receptor Inhibitor That Blocks the Growth of ER-Positive and ESR1-Mutant Breast Tumors in Preclinical Models. *Cancer Res* **76**, 3307-3318, doi:10.1158/0008-5472.CAN-15-2357 (2016).

34 Huang, K. L. *et al.* Proteogenomic integration reveals therapeutic targets in breast cancer xenografts. *Nature communications* **8**, 14864, doi:10.1038/ncomms14864 (2017).

35 Ellis, M. J. *et al.* Lower-dose vs high-dose oral estradiol therapy of hormone receptor-positive, aromatase inhibitor-resistant advanced breast cancer: a phase 2 randomized study. *JAMA* **302**, 774-780, doi:10.1001/jama.2009.1204 (2009).

36 Diggs-Andrews, K. A. *et al.* Sex Is a major determinant of neuronal dysfunction in neurofibromatosis type 1. *Ann. Neurol.* **75**, 309-316, doi:10.1002/ana.24093 (2014).

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